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Rapid and Long-Term Disappearance of CD4$^+$ T Lymphocyte Responses Specific for *Anaplasma Marginale* Major Surface Protein-2 (MSP2) in MSP2 Vaccinates following Challenge with Live *A. marginale*[1]

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In humans and ruminants infected with *Anaplasma*, the major surface protein 2 (MSP2) is immunodominant. Numerous CD4$^+$ T cell epitopes in the hypervariable and conserved regions of MSP2 contribute to this immunodominance. Antigenic variation in MSP2 occurs throughout acute and persistent infection, and sequentially emerging variants are thought to be controlled by variant-specific Ab. This study tested the hypothesis that challenge of cattle with *Anaplasma marginale* expressing MSP2 variants to which the animals had been immunized, would stimulate variant epitope-specific recall CD4$^+$ T cell and IgG responses and organism clearance. MSP2-specific T lymphocyte responses, determined by IFN-γ ELISPOT and proliferation assays, were strong before and for 3 wk postchallenge. Surprisingly, these responses became undetectable by the peak of rickettsemia, composed predominantly of organisms expressing the same MSP2 variants used for immunization. Immune responsiveness remained insignificant during subsequent persistent *A. marginale* infection up to 1 year. The suppressed response was specific for *A. marginale*, as responses to *Clostridium* vaccine Ag were consistently observed. CD4$^+$CD25$^+$ T cells and cytokines IL-10 and TGF-β1 did not increase after challenge. Furthermore, a suppressive effect of nonresponding cells was not observed. Lymphocyte proliferation and viability were lost in vitro in the presence of physiologically relevant numbers of *A. marginale* organisms. These results suggest that loss of memory T cell responses following *A. marginale* infection is due to a mechanism other than induction of T regulatory cells, such as peripheral deletion of MSP2-specific T cells. *The Journal of Immunology,* **2005, 174: 6702–6715.**

Pathogens in the genus *Anaplasma* express immunodominant outer membrane proteins with defined conserved and variable domains (1–6). Antigenic variation in *Anaplasma marginale* major surface protein 2 (MSP2)[3] and in the orthologous MSP2/p44 protein of *Anaplasma phagocytophilum*, results in evasion of the immune response and has been postulated to be responsible at least in part, for persistent infection in mammalian reservoir hosts (6–20). Although the acute phase of *A. marginale* infection peaks with levels of $\geq 10^9$ rickettsiae per milliliter of blood, persistent infection is characterized by recurrent subclinical cycles of rickettsemia that range from 10^3 to 10^7 organisms per milliliter (6, 7, 21–23). Each cycle of rickettsemia reflects the emergence of organisms that express antigenically variant MSP2 (7). Antigenic variation in MSP2, and in a related surface protein, MSP3, occurs by gene conversion of whole pseudogenes and small segments of pseudogenes into single expression sites, providing an efficient mechanism to generate the large number of variants seen during sequential cycles of persistent infection (8, 12, 13, 24).

The control of the sequential rickettsemic cycles during persistent infection is associated with development of a variant-specific IgG response and, in particular, IgG2 (7). In addition, MSP2 contains numerous MHC class II-restricted CD4$^+$ T cell epitopes in both the highly conserved N- and C-terminal domains as well as in the variant-specific central hypervariable region (HVR) (10, 11, 25, 26). This rich source of epitopes may serve to induce T cell help for generation of variant-specific Ab and control of rickettsemic cycles during persistent infection. In recent studies, we have used cattle immunized with purified MSP2 to define both the T and B lymphocyte epitopes in a specific set of MSP2 variants (10, 26). This allowed us to control the variants used for challenge in the context of the continual generation of new variants that occurs during actual infection. T cell epitopes were also recently mapped in 16 MSP2 vaccinates representing 10 different MHC class II *DRB3* alleles. IgG Ab responses were directed against epitopes predominantly located within the HVR, whereas CD4$^+$ T cell proliferative and IFN-γ responses were directed against multiple epitopes evenly distributed in the highly conserved and hypervariable regions (25). In the present study, we address stimulation and maintenance of anamnestic responses by specific MSP2 variants following infection. This study tested the hypothesis that challenge of cattle with *A. marginale* expressing MSP2 variants to which the animals had been immunized, would stimulate variant epitope-specific recall CD4$^+$ T cell and IgG responses and variant-specific

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[3] Abbreviations used in this paper: MSP2, major surface protein 2; HVR, hypervariable region; ODN, oligodeoxynucleotide; DPI, days postinfection; PCV, packed cell volume; URBC, uninfected bovine erythrocyte membrane; SFC, spot-forming cell; TCGF, T cell growth factor; AICD, activation-induced cell death.

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organism clearance. In this paper, data are presented that support rejection of this hypothesis and, in contrast, demonstrate a newly discovered immune modulation whereby Ag-specific T cell responsiveness is lost upon rickettsial challenge.

Materials and Methods

Animals and immunization

Cattle that were seronegative for *A. marginale* determined by an MSP5-specific competitive inhibition ELISA (27) were previously vaccinated with Vision 7 killed *Clostridium* spp. including *Clostridium chauvoei*, *Clostridium septicum*, *Clostridium novyi*, *Clostridium sordellii*, and *Clostridium perfringens types C&D* (Intervet) and 2–3 mo later with gel-purified native MSP2 (28). Four calves per group were immunized six times s.c. with 50 μg of MSP2 adsorbed in 2 mg of alum (Rehydragel, low viscosity sterile gel; Reheis) with either 10 μg of human IL-12 (kindly provided by Genetics Institute, Cambridge, MA) (animal nos. 01B71, 01B75, 01B76, and 01B82) or 1 mg of CpG oligodeoxynucleotide (ODN) 2006 (Oligos, Etc.) (animal nos. 01B78, 01B79, 01B81, and 01B87) as adjuvants (28). Negative control animals received alum and CpG ODN alone (animal nos. 01B73, 01B74, 01B84, and 01B89). All of the protocols in this study were reviewed and approved by the Washington State University Institutional Animal Care and Use Committee.

Challenge of immunized cattle

A splenectomized calf C949BL was inoculated i.v. with 0.2 ml of blood stabilate from calf C831BL infected with the Florida strain of *A. marginale*, the same batch of blood used for generating MSP2 for immunization, to ensure that the same MSP2 variants used for immunization were represented in the challenge inoculum. Twenty-six days postinfection (DPI), fresh blood was used for challenging the MSP2-immunized calves. To control the number of infectious organisms and to minimize contamination of bacterial proteins from dead organisms often found in frozen stabilate, the immunized and control calves were inoculated i.v. with $\sim 3 \times 10^3$ live organisms in 1 ml of PBS (pH 7.2). Microscopic examination of Wright-Giemsa-stained blood smears was performed daily to detect and quantify the level of *A. marginale* infection in the challenged animals. Packed cell volumes (PCVs) and rectal temperatures were also recorded daily.

PBMC and sera were collected from 5 mo following the last immunization (immediately before challenge), weekly thereafter until 2 mo following peak rickettsemia, and occasionally during persistent infection over the next year, and stored in liquid nitrogen (PBMC) or at −20°C (sera) for later use in T cell and Ab assays.

Sequencing of expression site msp2

Infected erythrocytes were washed three times in PBS with removal of the buffy coat after each wash, and genomic DNA was extracted from infected erythrocytes using the PureGene kit (Gentra Systems). The complement of *msp2* variants was analyzed in blood used for immunization (animal no. 831), blood used for challenge (animal no. 949), and blood from three immunized animals (nos. 76, 81, and 82) and two control animals (nos. 74 and 89) before, during, and after peak rickettsemia. Forward *msp2* primer (ATG AGT GCT GTA AGT AAT AGG AAG) or open reading frame 2 forward primer (TCC TAC CAA GCG TCT TTT CCC C) and *msp2* reverse primer (TTA CCA CCG ATA CCA GCA CAA) with *Taq* polymerase (Roche Applied Science) were used to amplify the *msp2* HVRs. All primer sequences correspond to the *msp2* operon sequence (GenBank accession no. AF200927), and all *msp2* sequences in the resulting PCR fragments correspond to the expression site (9). PCR was performed with genomic DNA and fragments cloned into the pCR4 TOPO vector (Invitrogen Life Technologies). Plasmid DNA was isolated, and inserts were sequenced in both directions with BigDye terminator chemistry on an ABI automated sequencer (PerkinElmer Applied Biosystems). Seventeen clones from blood used for immunization (animal no. 831) and 28 clones from blood used for infection (animal no. 949) were sequenced. In addition, ~30 clones were sequenced from each of the five animals, nos. 74, 76, 81, 82, and 89, at five time points spanning the period of peak rickettsemia. Thirty clones were attempted to ensure to a 95% confidence that all *msp2* variants expressed at least 10% of the time in the population were represented, according to the test of binomial proportions. Sequences of *msp2* from the earliest time point for animals nos. 74 and 76 were not obtained due to low numbers of organisms at that time. Sequences were compiled and analyzed using the Vector NTI (InforMax) software package. GenBank accession numbers for MSP2 variants F–Y are AY847664–AY847683.

Preparation of A. marginale*, MSP2 Ags, and MSP2-derived peptides*

Cryopreserved *A. marginale* Florida strain-infected bovine erythrocytes were prepared as previously described (28, 29). Native MSP2 was purified from sonicated *A. marginale* organisms subjected to preparative SDS gradient (10–20%) PAGE (30, 31). One lane of the gel with molecular markers was cut, transferred, and blotted with MSP2-specific mAb to orient the MSP2 on the gels. The MSP2 band was excised from multiple gels, and the protein was electroeluted from the gel fragments as described previously (31). Eluted protein was concentrated and dialyzed against PBS and purified a second time on preparative gels. MSP2 was verified by immunoblotting to be reactive with MSP2-specific mAb AnaF19E2 but not reactive with Abs that recognize *A. marginale* MSP1, MSP3, MSP4, and MSP5 (28). Imbricated 24- to 30-mer peptides that overlap by 10–20 aa and span the Florida strain MSP2 A variant sequence reported in GenBank (accession no. AY138954) were synthesized (10, 26). The MSP2 A variant was expressed from DNA clone 1-7, the most common variant transcript identified in the blood used for MSP2 immunization (10). The amino acid sequences of these peptides were reported previously (10, 26).

IFN-γ ELISPOT assays

Cryopreserved PBMC from immunized and control animals, obtained at the indicated days postchallenge, were analyzed for IFN-γ-secreting cells using an ELISPOT assay as described (28) with some modifications. After blocking and washing the plates, 0.5×10^6 PBMC were added in 100-μl volumes containing complete RPMI 1640 medium (11) alone, or with 10 and 1 μg/ml uninfected bovine erythrocyte membranes (URBC), *A. marginale* Florida strain homogenate, native MSP2, or MSP2-derived peptides (Table I). A mixture of 1.0 μg/ml PHA-P (Sigma-Aldrich), 0.01 ng/ml human IL-12 (Genetics Institute), and 0.5 ng/ml human IL-18 (PeproTech), shown to stimulate high levels of IFN-γ in bovine PBMC (32), was used as a positive control. After incubation for 40 h at 37°C, the plates were washed, developed, and dried overnight. Spots were visualized using an ELISPOT reader (Cell Technology) and AID 2.9 software (AutoImmun Diagnostika). For each PBMC sample, the mean number of spots in the negative control wells was subtracted from the mean number of spots in test wells to determine the mean number of *A. marginale* MSP2-specific IFN-γ-secreting cells or spot-forming cells (SFC). Results are presented as the mean number of SFC per 10^6 PBMC.

Lymphocyte proliferation assays

Proliferation assays were conducted in replicate wells of round-bottom 96-well plates (Costar) for 6 days, essentially as described (26, 33). PBMC (2×10^5) isolated at the same time as for the ELISPOT assay were cultured for 6 days in triplicate wells with dilutions of 10, 1, and 0.1 μg/ml Ags identical with those used in the ELISPOT assay. In addition to the PHA, IL-12, and IL-18 mixture, bovine T cell growth factor (TCGF) at a final dilution of 10% was included as a positive control for proliferation. The PBMC were radiolabeled for the last 18 h of culture with 0.25 μCi of [^{3}H]thymidine (DuPont, New England Nuclear) and harvested onto glass filters, and radionucleotide incorporation was determined using a Betaplate 1205 liquid scintillation counter (Wallac). Results are presented as the mean cpm of triplicate cultures ± 1 SD.

MSP2-specific IgG titers

ELISAs were used to determine MSP2-specific IgG1 and IgG2 titers as described (28) with the following changes. Sera (100 μl) from the 12 study cattle diluted from 1/10 to 1/100,000 were added per well, followed by 100 μl/well of 1 μg/ml bovine IgG1-specific mAb BIG 715A (WSU Monoclonal Center) or a 1/100 dilution of bovine IgG2-specific mAb K192 4F10 (Serotec). These mAb concentrations bound to equivalent amounts of purified bovine IgG1 and IgG2 (Serotec). The OD_{405} were determined using a Titertek Multiscan MCC/340 microplate reader (MTX Lab Systems).

Cytokine ELISAs

IL-10, TGF-β1, and IL-4 ELISAs were used to analyze supernatants from PBMC grown in culture for 72 h with 5 μg/ml MSP2 for the secretion of cytokines. The IL-10 ELISA was done as previously described (34) with the following modifications. Black 96-well microplates (Porvair) were incubated overnight at 4°C with capture mAb CC318 at 6 μg/ml in coating buffer. All additional incubations were at room temperature. The plates were washed with PBS containing 0.5% Tween 20 (PBST) and blocked with PBST containing 1% BSA for 1 h. Following blocking, 100 μl of cell culture supernatants were added to each well and incubated for 1 h. Following six washes with PBST, 100 μl of 2 μg/ml biotin-labeled secondary

Table I. *Prevalence of* A. marginale msp2 *variants*

Number and Letter Designation of *msp2* Variants Sequenced							
Animal 831	Animal 949		Animal 82	Animal 76	Animal 81	Animal 74	Animal 89
Immunization group[a]	Challenge group[b]	DPI	MSP2 + IL-12 group[c]	MSP2 + IL-12 group	MSP2 + CpG group	CpG only group	CpG only group
10-A	**19-A**	24	**6-A**		**7-A**		**10-A**
4-C	**3-D**		**1-D**		**1-D**		1-Q
1-D	**1-F**		**1-G**		**1-F**		
1-E	**1-G**				**2-G**		
1-F	1-H						
1-G	**1-I**						
1-I	2-O						
		31	**20-A**	**18-A**	**28-A**	**16-A**	**18-A**
			2-D	**1-F**	**1-D**	**12-D**	**5-D**
			1-C	**11-G**	**1-G**		**3-E**
			6-G		1-K		**1-I**
							1-K
							2-Q
							1-W
		38	**12-A**	**21-A**	**6-A**	**28-A**	**3-A**
			1-B	**7-G**	**1-C**	**6-D**	**2-C**
			1-C		**3-D**		**12-D**
			2-D		**1-F**		**3-E**
			10-G		**9-G**		**2-G**
			1-J		**2-I**		2-K
			1-K		2-K		1-R
			1-Q		4-Q		1-U
					1-R		1-W
		45	**9-A**	**28-A**	**15-C**	**5-A**	**1-F**
			6-B	**2-E**	**2-G**	**27-D**	**16-G**
			1-C	**1-F**	1-R		5-K
			2-D	**3-G**			2-N
			6-G				3-R
			2-J				
			1-K				
		52	1-B	**16-A**	**4-C**	**5-A**	**2-C**
			22-G	**1-C**	**2-D**	**1-C**	**1-D**
			1-L	**2-D**	**6-G**	**20-D**	**12-G**
			1-M	**5-E**	2-N	**2-G**	6-N
			2-N	**2-F**	2-O	1-V	1-R
			1-O	**4-G**	2-Q		1-V
			2-R	1-P	3-R		4-Y
					1-T		

[a] *msp2* variants and relative number sequenced from blood used to purify MSP2 protein for immunization.
[b] *msp2* variants and relative number sequenced from blood used for challenge of eight immunized and four control animals.
[c] *msp2* variants and relative number sequenced from blood of challenged animals. Bolding indicates variants identical to *msp2* variants present in the blood used to purify the MSP2 for immunization.

mAb CC320, was added to each well and incubated for an additional 1 h. The plates were washed six times with PBST, and 100 μl of the Super Signal ELISA Femto Maximum Sensitivity substrate (Pierce) was added, and the plates were evaluated within 5 min. The relative light unit value was read on Betaplate 1205 liquid scintillation counter and luminometer (Wallac).

The IL-4 ELISA was performed using the same protocol described for IL-10 with the use of bovine IL-4-specific mAbs. mAb CC314 was used for coating, and biotinylated mAb CC313 was used for detection (61).

The TGF-β1 ELISA was performed per manufacturer's protocol no. TB196 (Promega). Samples were diluted 1/16 in 1× TGF-β1 sample buffer and then acidified for 15 min by addition of 1.0 μl of 1 N HCl/50 μl of sample. The samples were neutralized by adding 1.0 μl of 1 N NaOH/50 μl of sample until the sample pH was 7.6. Immulon II 96-well ELISA microplates (Dynax Technologies) were coated with 100 μl of 1 μg/ml anti-human TGF-β1 "coat mAb" in carbonate coating buffer (0.025 M sodium bicarbonate, 0.025 M sodium carbonate, pH to 9.7) overnight at 4°C. The plates were emptied and blocked with 270 μl of 1× TGF-β1 blocking buffer for 35 min at 37°C. After washing three times with PBST, 100 μl of sample supernatants was added and incubated at room temperature for 90 min while shaking. After washing six times with PBST, the plates were incubated with "anti-TGF-β1 pAb" at room temperature for 2 h, while shaking followed by six additional washes with PBST. To each well, 100 ml of TGF-β1 HRP conjugate was added and incubated for 2 h at room temperature while shaking. After washing six times with PBST, 100 μl of tetramethylbenzidine solution was added to each well and incubated for 15 min. To each well, 100 μl of 1 N HCl was added, and the OD_{450} was determined.

Flow cytometric analysis

PBMC were stained for surface expression of CD4 (mAb IL-A11), CD8 (mAb 7C2B), and $\gamma\delta$ TCR (TCR1) (mAb GB21A). Secondary goat anti-mouse isotype-specific Abs conjugated with PE were used for visualizing CD4, CD8, and $\gamma\delta$ TCR1-staining cells. For two-color analysis, CD25-specific mAb CACT 116A and goat anti-mouse isotype-specific, FITC-conjugated secondary Abs were used. All primary mAb were obtained from the Washington State University Monoclonal Antibody Center. Secondary Abs were obtained from Caltag Laboratories. Samples were analyzed using a FACSCalibur flow cytometer (BD Biosciences). At least 5×10^5 cells were used for staining, and 10,000 cells were acquired using BD Biosciences CellQuest software.

Coculture of responding and nonresponding PBMC

Proliferation assays mixing responding (pre-peak rickettsemia) and nonresponding (peak rickettsemia) PBMC were performed as described above with the following changes. A fixed number of responding cells numbering either 0.5×10^5 PBMC/well or 1×10^5 PBMC/well were cultured with varying numbers of nonresponding PBMC, which were 4-, 2-, 1-, 0.5-, and 0-fold the number of responding cells. As a control for total numbers of cultured cells, responding PBMC were cultured at a number equal to that of the total number of responding plus nonresponding PBMC.

Culture of enriched CD4$^+$ T cells with naive APC

CD4$^+$ T cells were positively selected from responding PBMC and nonresponding PBMC taken at the peak of rickettsemia from cattle 71 and 76, and tested for Ag-specific proliferation using APC prepared from noninfected cattle that expressed a half-matched, homozygous MHC class II haplotype. PBMC frozen at 1 wk postchallenge from animal 71 and at 2 wk prechallenge from animal 76 (responding cells) and at 6 wk postchallenge from animal 71 and at 5 wk postchallenge from animal 76 (nonresponding cells) were used. CD4$^+$ T cells were positively selected from thawed cells following incubation with anti-CD4 mAb ILA-11 and goat anti-mouse IgG-coated *MACS* MicroBeads beads following the manufacturer's instructions (Miltenyi Biotec). Following several washes in complete RPMI 1640, the CD4$^+$ T cells (1×10^5 cells/well) were cultured overnight in U-bottom 96-well plates in 100 μl of complete RPMI 1640 with 1×10^5 irradiated PBMC from the donor animal half-matched at MHC class II. The next day, the plates were centrifuged at $900 \times g$, and ~75 μl of supernatant was replaced by fresh medium to remove residual anti-CD4 mAb. Ag was then added to triplicate wells, and the cells were incubated for an additional 4 days and pulsed with [^{3}H]thymidine during the last 18 h of culture. Cells were harvested and counted, and the data are presented as mean cpm ± 1 SD. FACS analysis of the CD4$^+$ positively selected cells revealed a mean of 78% CD4$^+$ T cells, 18% CD8$^+$ cells, and 4% CD14$^+$ cells.

MHC class II *DRB3* alleles were defined by PCR-restriction fragment length polymorphism of exon 2 and by sequencing the *DRB3* cDNAs (25, 35). APC from cattle 98B61 (*DRB3 8/8*) and 201 (*DRB3 22/22*) were used, respectively, to present Ag to CD4$^+$ T cells from MSP2-immunized cattle 71 (*DRB3 8/14*) and 76 (*DRB3 22/21*).

Inhibition of lymphocyte viability and proliferation by A. marginale

Initial bodies were purified essentially as described (29). Blood from an *A. marginale*-infected animal with 23% infected erythrocytes was washed five times with PBS and centrifuged at $30{,}000 \times g$ for 30 min after each wash. Infected erythrocytes were resuspended in PBS containing Complete Mini protease inhibitors following manufacturer's instructions (Roche). *A. marginale* organisms were released from infected erythrocytes by sonication with the Sonifier Cell Disruptor 350 (VWR Scientific) at an output control setting of 4 at 100% duty cycle, followed by centrifugation at $1500 \times g$ for 15 min. The pelleted organisms were resuspended in PBS and stored at −20°C. Protein determination revealed that this preparation contained 16 mg/ml protein. Cryopreserved PBMC (2×10^5 cells/well) from an *A. marginale*-naive donor cow were thawed, washed, and cultured for 3 days with 10% TCGF with or without *A. marginale* using ~3–50 $\times 10^6$ infected erythrocyte equivalents per milliliter. The cells were either pulsed with [^{3}H]thymidine, harvested, and counted, or triplicate wells were pooled and cell viability was determined by trypan blue dye exclusion. Results are presented as mean cpm + 1 SD of triplicate cultures or mean number of viable cells/milliliter.

Data analysis and statistics

All statistical tests were done with Number Cruncher Statistical Software (NCSS2001), version 2.00.0185. The proportion of the *msp2* A variant in the organisms used to prepare MSP2 for immunization and for challenge was compared by the Fisher Exact test. Clinical parameters of the immunized groups were compared by Kruskal-Wallis rank sum analysis with the control group ($\alpha = 0.05$). Correlation of IgG titers with clinical parameters was determined using multivariate linear regression and Spearman rank correlation. One-way ANOVA with Bonferonni correction for multiple comparisons ($\alpha = 0.05$) was used to determine significant ELISPOT and proliferation responses as compared with medium, and to determine significant differences in CD25$^+$ T lymphocyte populations. Paired two-tailed *t* tests were used to determine significance differences in cytokine levels between responding and nonresponding PBMC.

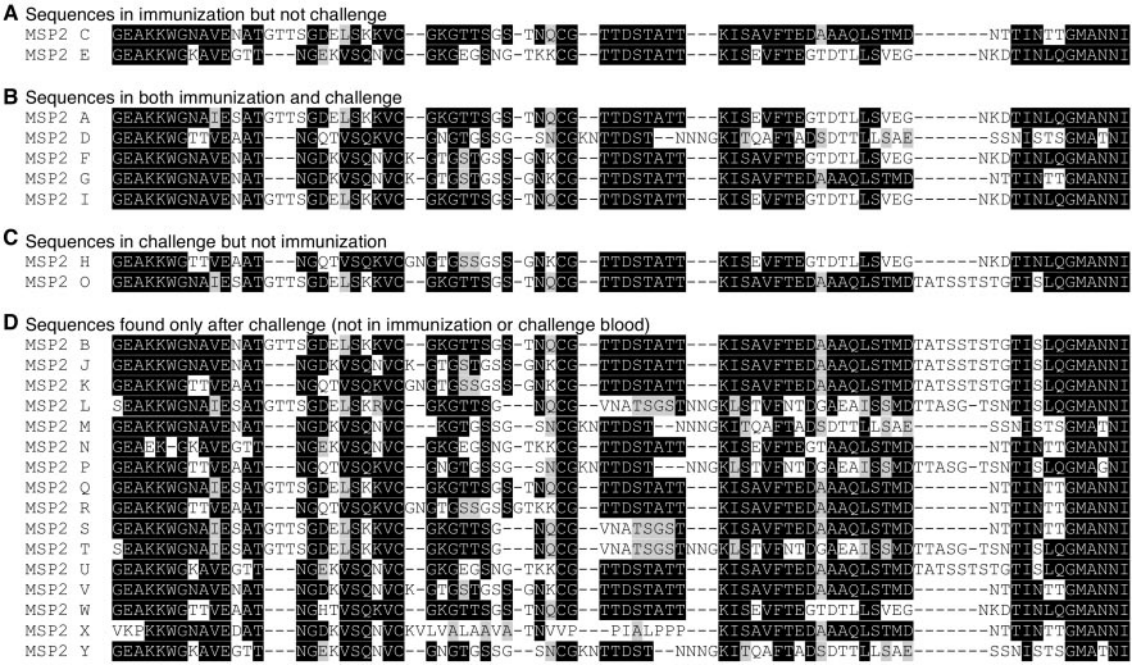

FIGURE 1. Predicted amino acid sequences for *msp2* variants identified in this study. *msp2* genes were cloned from blood used to purify MSP2 for immunization (*A* and *B*), from blood used for challenge of the vaccinates and control calves (*B* and *C*), and from blood of five of the challenged animals at the indicated time points before, during, and after peak rickettsemia (*D*). *A* contains *msp2* variant sequences found in the immunogen, but not the challenge blood. *B* contains *msp2* variant sequences common to both immunogen and challenge blood. *C* contains *msp2* variant sequences found in the challenge blood, but not the immunogen. All sequences begin at aa 183 corresponding to the *msp2* 11.2 genomic DNA clone (15).

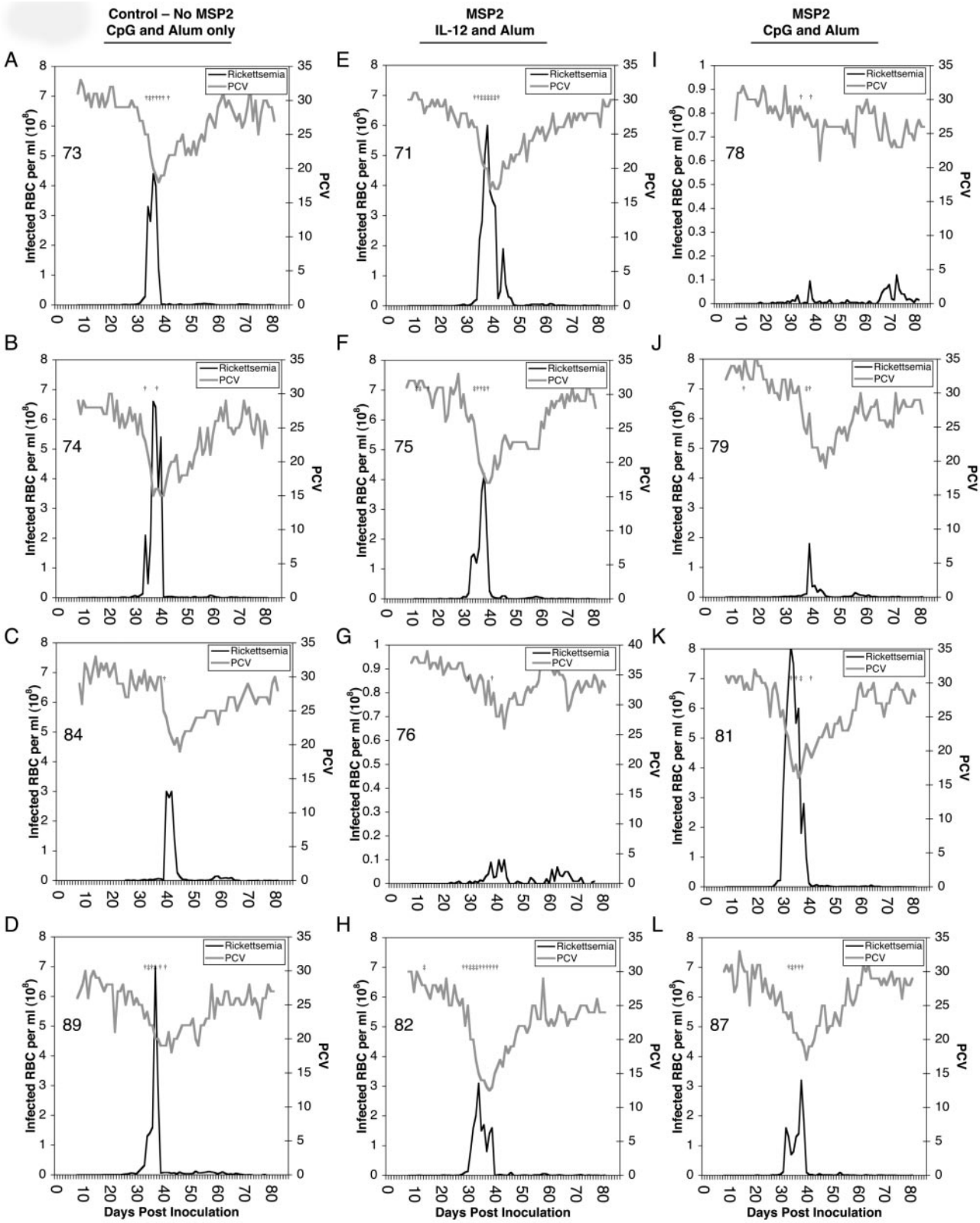

FIGURE 2. Rickettsemias and PCVs of eight immunized and four control cattle during acute anaplasmosis. Cattle were challenged 5 mo postimmunization with ~3000 live *A. marginale* organisms. Rickettsemias and PCVs were measured daily for control animals immunized with alum and CpG ODN 2006 only (*A–D*) and animals immunized with MSP2, alum, and either IL-12 (*E–H*) or CpG ODN 2006 (*I–L*). Animal numbers are indicated on each panel. Rickettsemia (*left axis*) is reported as number of infected erythrocytes per milliliter of whole blood as determined by microscopic evaluation of daily blood smears. Daily PCV (*right axis*) was used to evaluate anemia during infection. †, Indicates days when the temperature was >102°F. ‡, Indicates days when the temperature was >103°F.

Results

Comparison of MSP2 variants in the MSP2 immunogen and challenge organisms

The relationship between *msp2* variants in the *A. marginale*-infected blood used as a source of MSP2 immunogen and the *msp2* variants expressed by the organisms in the blood used for challenge was determined by sequencing the *msp2* single expression site in these organisms (Fig. 1). In the organisms used as a source of preparing MSP2, 10 of the 19 *msp2* clones sequenced were variant A, 4 were variant C, and 5 additional minor variants were each represented by a single clone (Table I). The *A. marginale* used for challenge was also composed predominantly of the *msp2* A variant (19 of 28 clones) and contained 6 additional minor variants (Table I). Although amplification of DNA by PCR to determine the relative frequency of *msp2* variants may introduce bias, previous studies have shown a positive correlation in relative levels of *msp2* variants amplified by PCR with levels of *msp2* expression site genomic DNA, *msp2* mRNA, and MSP2 protein (12, 36). In the present study, no statistically significant difference was found in the frequency of the *msp2* A variant in the organisms used to prepare native MSP2 for immunization and those used for challenge ($p = 0.365$). The predicted amino acid sequences of the HVRs of all *msp2* variants are shown in Fig. 1, *A–C*. Variants A, B, C, D, and E have been previously reported (GenBank accession nos. AY138954–AY138958) (10). Two minor *msp2* variants (H and O) were present in the challenge organisms that were not found in the MSP2 immunogen (Fig. 1*A*), and two minor variants (C and E) were present in the immunogen but not detected in the challenge organisms (*C*).

Challenge of MSP2-immunized cattle with live A. marginale

Following challenge with $\sim 10^3$ live *A. marginale* organisms, all animals had microscopically measurable rickettsemia by 25 DPI (Fig. 2). Ten of the 12 animals had levels of rickettsemia exceeding 10^8 infected erythrocytes per milliliter of blood at the peak of infection, ~5–6 wk postchallenge. The remaining two animals, one from each vaccination group, had $>10^7$ infected erythrocytes per milliliter of blood. Clinical parameters including peak rickettsemia levels, days to peak rickettsemia, days to detection of rickettsemia, days to 10^8 infected erythrocytes per milliliter, and the duration of the rickettsemia peak varied from animal to animal, but there were no significant differences in these parameters of infection between immunized and control groups (Fig. 2).

Analysis of msp2 variants that arose following A. marginale *challenge*

One possible explanation for the lack of protection in MSP2 vaccinates is that MSP2 variants unique to the challenge inoculum could have evaded the immune response and expanded rapidly in the immunized animals, causing acute rickettsemia. To examine this possibility, ~30 *msp2* variants were cloned and sequenced from three immunized and two control cattle at five time points spanning the peak and resolution of acute rickettsemia (Table I). The relative frequencies of the *msp2* variants identified during acute rickettsemia in the five animals are shown in Table I. Fig. 1*D* shows only sequences of unique *msp2* variants that arose during acute rickettsemia in the five animals examined. At 24 DPI, which was before consistent microscopic detection of rickettsia, the *msp2* A variant was the predominant variant in each animal, regardless of immunization status (Table I). At 31 DPI, the *msp2* A variant still predominated in the blood of all five animals tested. Interestingly, one vaccinate (animal 76) and one control (animal 74) had not completely cleared the *msp2* A variant by 52 DPI (Table I), when rickettsemia had dropped. Even though the other three animals succeeded in clearing the *msp2* A variant by 52 DPI, the predominant variants throughout all time points were *msp2* variants present in the immunogen (Table I). Thus, the acute rickettsemia in MSP2 vaccinates was apparently not due to the emergence of minor or novel MSP2 variants.

MSP-specific IgG responses

To determine whether anamnestic MSP2-specific IgG responses occurred following challenge of immunized animals, IgG1 and IgG2 titers were measured before challenge and at several time points thereafter (Table II). Before challenge, all immunized, but not control, animals had measurable IgG1 titers, and all but one had measurable IgG2 titers. After challenge, the immunized animals developed higher titers than the controls. However, the Ab titers in both immunized and control animals peaked at the time of peak rickettsemia, then tapered off over 2 mo following resolution

Table II. A. marginale *MSP2-specific IgG1 and IgG2 titers in the sera of immunized and control animals before and during acute anaplasmosis*

	IgG1 Titer[a]				IgG2 Titer[a]			
	Prechallenge	Prepeak	Peak	Postpeak	Prechallenge	Prepeak	Peak	Postpeak
Group and Calf No.	0 DPI	21–24 DPI	36–38 DPI	92–97 DPI	0 DPI	21–24 DPI	36–38 DPI	92–97 DPI
MSP2 + IL-12 + alum								
71	100	100	10,000	1,000	10	10	1,000	500
75	10	10	5,000	1,000	10	10	10	10
76	100	100	10,000	1,000	500	500	10,000	1,000
82	1,000	5,000	50,000	10,000	10	100	10	10
MSP2 + CpG + alum								
78	100	100	100	1,000	100	100	100	1,000
79	1,000	500	10,000	1,000	100	100	100	100
81	100	500	1,000	500	0	0	0	0
87	10	1,000	5,000	1,000	10	10	10	10
CpG + alum only								
73	0	10	5,000	100	0	0	0	0
74	0	10	100	100	0	0	0	10
84	0	10	100	100	0	0	10	10
89	0	10	1,000	500	0	0	0	0

[a] *Titers are expressed as the reciprocal of the highest dilution of serum that reacted by ELISA.*

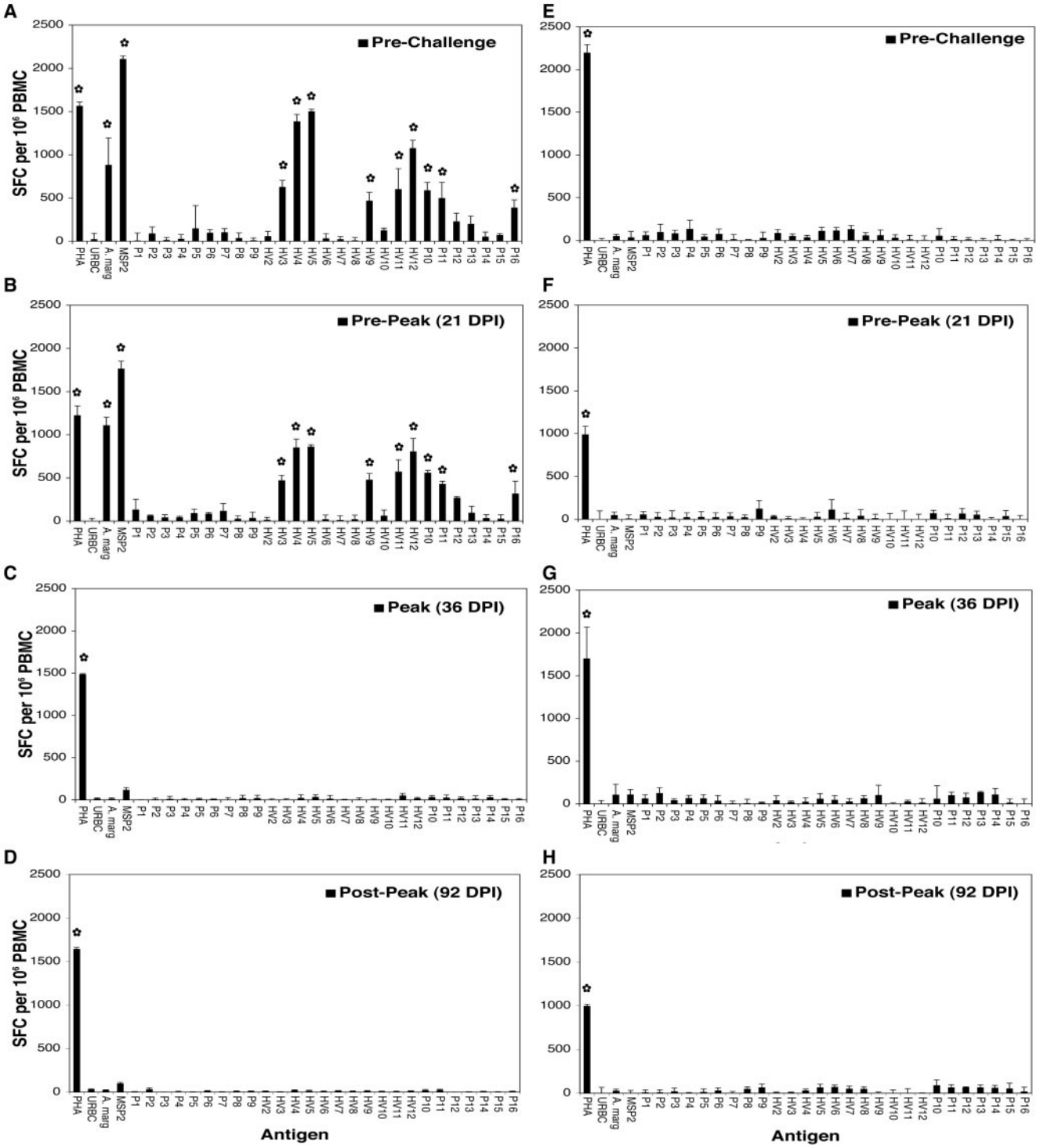

FIGURE 3. Comparison of $CD4^+$ T cell epitopes recognized by cattle before and following *A. marginale* challenge by IFN-γ ELISPOT assay. Representative data from PBMC from animal 71 from the IL-12 adjuvant group (*A–D*) and animal 73 from the control group (*E–H*) are presented. PBMC were assayed for responses to the mixture of PHA plus IL-12 plus IL-18 (PHA), and 10 μg/ml *A. marginale* lysate, whole MSP2, or overlapping peptides spanning MSP2. Assays were done simultaneously with cryopreserved PBMC obtained at the indicated time points: before challenge (*A* and *E*), following challenge, but before peak rickettsemia (*B* and *F*), at peak rickettsemia (*C* and *G*), and 2 mo following peak rickettsemia (*D* and *H*). Data are presented as the mean number of SFC + 1 SD of triplicate cultures with Ag minus the mean number of SFC of triplicate cultures with medium, and are reported as mean number of SFC/10^6 PBMC.

of clinical disease, and remained at or higher than prechallenge levels, which is consistent with persistent infection. Also, as seen previously (28), IgG1 levels overall were significantly higher than IgG2 levels throughout acute infection. These results also show that there was no correlation of MSP2-specific IgG1 and IgG2 Ab titers and level of rickettsemia; however, the cattle with the lowest levels of rickettsemia were the only animals with prechallenge IgG2 titers of ≥100.

Comparison of MSP2-specific CD4$^+$ T cell responses before and after challenge by IFN-γ ELISPOT assay

To determine whether challenge evoked an anamnestic CD4$^+$ T cell response specific for conserved or variable MSP2 epitopes in MSP2 vaccinates, IFN-γ ELISPOT assays were performed using PBMC obtained before and following challenge. *A. marginale,* native MSP2, and overlapping peptides spanning the predominant MSP2 A variant were used for stimulation of PBMC. We previously demonstrated that CD4$^+$ T cells in PBMC of these MSP2-immunized animals were the responding cells (25). Assays for each individual were done simultaneously using PBMC obtained immediately before challenge (5 mo after immunization), 21–23 DPI (before peak rickettsemia), 36–38 DPI (peak rickettsemia), and 92–94 DPI (post-peak rickettsemia).

Before peak rickettsemia, the IFN-γ ELISPOT responses to MSP2-derived peptides were overall similar in magnitude to those seen following MSP2 immunization in all MSP2-immunized animals, suggesting that challenge did not boost the pre-existing response (Fig. 3 and Table III). What was more interesting, however, was the finding that Ag-specific CD4$^+$ T cell responses determined at the time of peak rickettsemia and at all time points thereafter measured in individual animals for 7 mo to 1 year postchallenge, were severely decreased to background or near background levels for all animals and Ags tested (Figs. 3 and 4). Furthermore, none of the four control animals generated a significant CD4$^+$ T cell response to *A. marginale*, MSP2, or any of the MSP2-derived peptides at the time points examined postchallenge for up to 3 mo (Fig. 3). In contrast to the MSP2-specific response, similar levels of response to the mixture of PHA, IL-12, and IL-18 were maintained throughout the course of infection. These results were obtained in at least two independent assays. Fig. 3 illustrates these results for PBMC obtained from immunized animal 71 and control animal 73 at two time points before peak rickettsemia (Fig. 3, *A* and *B*; and *E* and *F*, respectively), at peak rickettsemia (*C* and *G*), and following peak rickettsemia (*D* and *H*). Table III summarizes the ELISPOT assay results for all animals, presenting only data for those conserved and hypervariable region peptides that induced strong responses before peak rickettsemia in the majority of animals. Antigenic variation in MSP2 does not explain the sudden loss of response, because the decrease in CD4$^+$ T cell responses included those responses to peptides from both conserved as well as HVRs.

Comparison of IFN-γ ELISPOT and proliferation assays to MSP2 and to Clostridium *vaccine Ag*

We recently showed a significant correlation between proliferation and IFN-γ ELISPOT responses by PBMC from these MSP2 vaccinates to *A. marginale*, MSP2, and MSP2-derived peptides (25).

Table III. *Comparison of IFN-γ-secreting CD4$^+$ T lymphocytes in PBMC before and after challenge*: *the percentage of SFC in PBMC obtained postchallenge relative to the number of SFC obtained prechallenge in the animals*[a]

		IL-12 Animal Group				CpG Animal Group			
Antigen		Animal 71	Animal 75	Animal 76	Animal 82	Animal 78	Animal 79	Animal 81	Animal 87
A. marginale	Prechallenge (no. of SFC per 10^6 PBMC)[b]	**587**	**198**	125	**829**	**150**	**231**	73	**723**
	Prepeak (%)[c]	**189**	**120**	102	**33**	**110**	**316**	100	**50**
	Peak (%)	2	**80**	**220**	0	41	6	24	3
	Postpeak (%)	4	53	74	17	36	17	124	13
MSP2	Prechallenge (no. of SFC per 10^6 PBMC)	**1404**	**933**	**663**	**855**	**752**	**527**	**181**	**261**
	Prepeak (%)	**125**	**93**	**105**	**123**	**97**	**185**	**96**	**150**
	Peak (%)	8	**32**	12	8	47	5	29	13
	Postpeak (%)	7	4	8	16	11	0	0	8
P7	Prechallenge (no. of SFC per 10^6 PBMC)	67	**343**	66	**935**	148	**188**	16	126
	Prepeak (%)	174	**79**	**311**	**87**	**106**	**190**	271	**235**
	Peak (%)	1	13	0	0	13	2	0	5
	Postpeak (%)	0	3	15	12	0	0	129	0
HV3	Prechallenge (no. of SFC per 10^6 PBMC)	**417**	**417**	**555**	**769**	**180**	57	135	9
	Prepeak (%)	**112**	**133**	**125**	**118**	**123**	**309**	36	262
	Peak (%)	0	0	3	1	**89**	24	17	300
	Postpeak (%)	1	0	5	8	21	74	29	108
HV4	Prechallenge (no. of SFC per 10^6 PBMC)	**921**	**601**	**173**	**647**	**311**	**191**	**231**	63
	Prepeak (%)	**92**	**119**	**160**	**90**	**162**	**102**	21	6
	Peak (%)	2	0	15	10	**118**	2	13	26
	Postpeak (%)	2	0	10	19	8	24	28	10
P10	Prechallenge (no. of SFC per 10^6 PBMC)	**391**	**649**	47	**1027**	**210**	60	82	55
	Prepeak (%)	**142**	**112**	120	**51**	**164**	180	128	**305**
	Peak (%)	8	24	17	3	0	18	7	0
	Postpeak (%)	5	10	64	8	6	0	116	22
P13	Prechallenge (no. of SFC per 10^6 PBMC)	131	**269**	**594**	**1197**	**510**	71	97	105
	Prepeak (%)	72	**109**	**115**	**56**	**123**	75	20	**275**
	Peak (%)	9	24	9	8	10	9	12	0
	Postpeak (%)	2	0	14	7	7	25	0	2

[a] Cryopreserved PBMC from immunized or control animals were stimulated for 40 h with 10 μg/ml *A. marginale* lysate, whole MSP2, or MSP2-derived peptides in the IFN-γ ELISPOT assay. Results are presented as mean number of SFC per 10^6 PBMC (prechallenge) after subtracting the mean number of SFC in cultures with medium. Responses ≥150 SFC per 10^6 PBMC are considered significant and are indicated in bold.

[b] Prechallenge—mean number of SFC per 10^6 PBMC stimulated by the indicated Ag. Peptides P7, P10, and P13 are from conserved regions, and HV3 and HV4 are from the central variable region of MSP2.

[c] Postchallenge—the percentage of the prechallenge number of SFC per 10^6 PBMC.

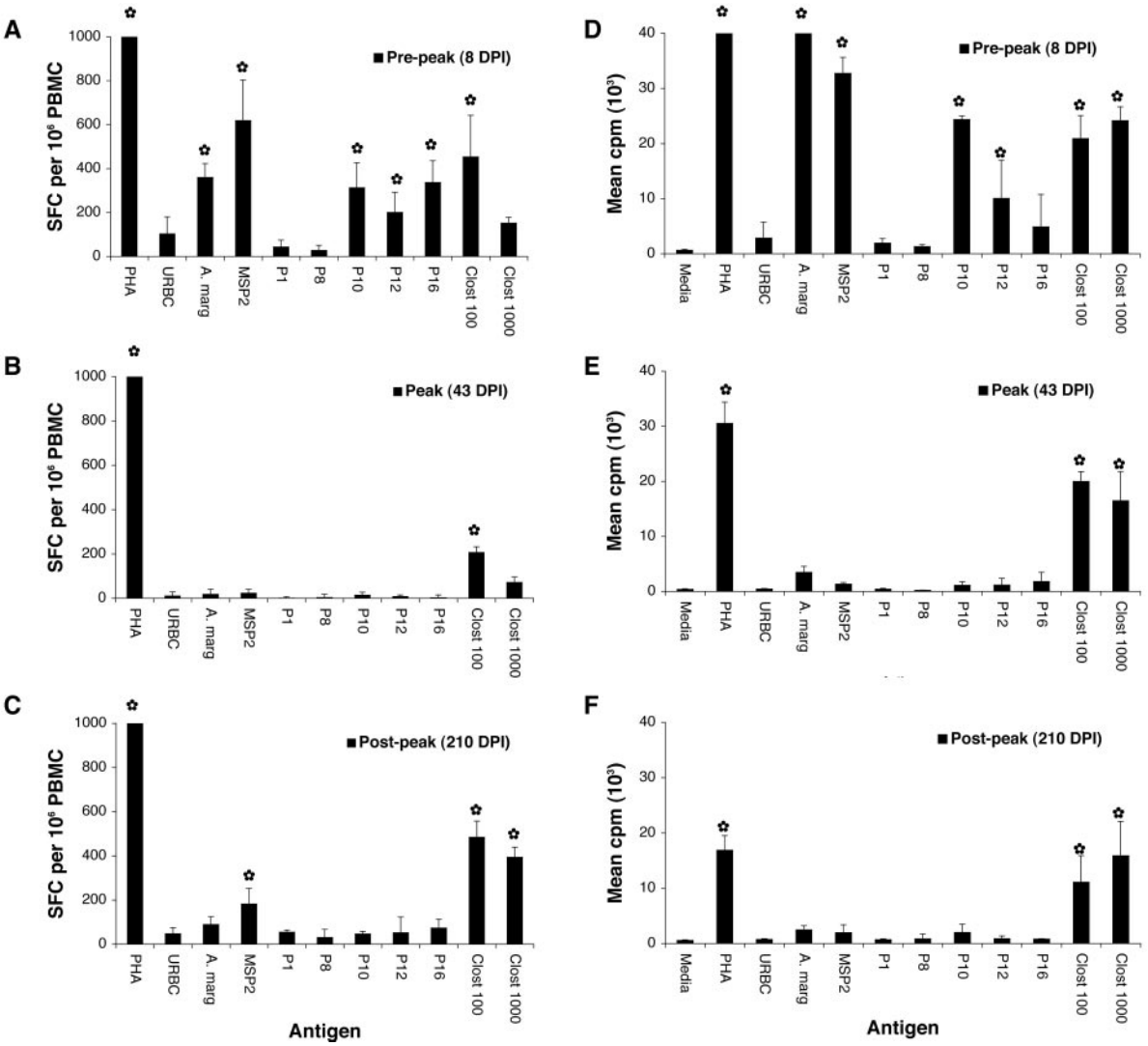

FIGURE 4. Comparison of IFN-γ ELISPOT and proliferation assays using PBMC obtained before and following *A. marginale* challenge. PBMC were obtained from animal 71 at the indicated time points, cryopreserved, and assayed simultaneously for IFN-γ-secreting cells (*A–C*) or proliferation (*D–F*). PBMC were stimulated with PHA plus IL-12 plus IL-18 (PHA), or 10 μg/ml *A. marginale* lysate, whole MSP2, and MSP2-derived peptides, or *Clostridium* vaccine Ag diluted 1/100 and 1/1000. Data are presented as the mean number of SFC + 1 SD of triplicate cultures with Ag minus the mean number of SFC of triplicate cultures with medium, and are reported as the mean number of SFC/10^6 PBMC (*A–C*), or as the mean cpm + 1 SD of triplicate cultures (*D–F*).

Nevertheless, to address the possibility that following *A. marginale* infection, the response changed from a predominant Th1-like response to a predominant Th2-like response that could not be detected by the IFN-γ ELISPOT assay, proliferation and IFN-γ ELISPOT assays were conducted simultaneously with the same aliquots of cells. Consistent with the results using the ELISPOT assay, proliferative responses to *A. marginale*, MSP2, and all MSP2-derived peptides were severely decreased in all eight vaccinates at the peak of infection and thereafter, whereas the response to the PHA, IL-12, and IL-18 was always observed (representative data for MSP2-immunized animal 71 are shown in Fig. 4, *D–F*).

To further determine whether the impaired T cell response to *A. marginale* and MSP2 was Ag specific or reflected a generalized immune suppression, *Clostridium* spp. Ag, which was used to vaccinate the calves before MSP2 immunization, was also included in the assays. Unlike the responses to *A. marginale* and MSP2, the response to *Clostridium* Ag was significant at all time points (Fig. 4). In addition, no significant responses to MSP2-derived peptides were seen (Fig. 4, *E* and *F*). Similar results were obtained for all immunized animals tested at various time points up to 7–12 mo postinfection (data not presented), and suggest that the impaired response to MSP2 is long-lived and does not reflect a generalized immune suppression.

Quantitation of $CD25^+CD4^+$ T cells by FACS

Regulatory T cells have not been defined in cattle. Nevertheless, to evaluate the potential role of $CD25^+CD4^+$ T regulatory cells in the loss of MSP2-specific responses following challenge, $CD25^+CD4^+$ T cells were analyzed in PBMC from immunized and control animals by two-color flow cytometry (Table IV). Although the differences in the percentage of $CD25^+CD4^+$ T cells in PBMC varied between individual animals, when data for all animals were compared, no significant differences in the percentage of $CD25^+CD4^+$ T cells, expressed as either a percentage of total $CD4^+$ T cells or of total $CD25^+$ T cells, were observed during the course of infection (data not shown).

Table IV. *Percentage of CD25$^+$CD4$^+$ T cells in PBMC from cattle during acute anaplasmosis: percentage of cell types in total PBMC as determined by flow cytometric analysis*[a]

Immunization Group	Animal	Time	CD4$^+$ and CD25$^+$	CD3$^+$ and CD4$^+$	CD3$^+$ and CD25$^+$
IL-12	71	Prepeak[b]	ND	15	17
Alum		Peak	ND	7	4
MSP2		Postpeak	ND	11	15
	75	Prepeak	3	16	14
		Peak	2	16	10
		Postpeak	5	19	20
	76	Prepeak	4	22	11
		Peak	2	25	3
		Postpeak	3	25	6
	82	Prepeak	1	17	7
		Peak	1	13	5
		Postpeak	2	2	4
CpG	78	Prepeak	3	13	2
Alum		Peak	1	11	4
MSP2		Postpeak	3	15	12
	79	Prepeak	1	14	2
		Peak	1	8	3
		Postpeak	1	14	5
	81	Prepeak	2	13	13
		Peak	2	11	4
		Postpeak	7	23	4
	87	Prepeak	1	13	3
		Peak	1	9	5
		Postpeak	3	15	5
CpG	73	Prepeak	2	16	11
Alum		Peak	1	11	5
No MSP2		Postpeak	2	21	9
	74	Prepeak	1	17	5
		Peak	1	13	5
		Postpeak	1	16	4
	84	Prepeak	ND	ND	ND
		Peak	ND	ND	ND
		Postpeak	ND	ND	ND
	89	Prepeak	18	11	20
		Peak	1	11	2
		Postpeak	3	23	8
No immunization nor infection	80		3	17	3

[a] FACs analysis was done using frozen PBMC from three time points following challenge with 3000 live *A. marginale* organisms. mAb ILA11 (CD4) and CACT116A (CD25), and MM1A (CD3) were used for dual color staining.
[b] Time points are prepeak rickettsemia, peak rickettsemia (Peak), and postpeak rickettsemia.

Comparison of IL-10, TGF-β1, and IL-4 production by responding and nonresponding PBMC

IL-10 and TGF-β1 are cytokines produced by subsets of regulatory T cells in mice and humans (37–40). Therefore, to investigate a potential role of these cytokines either in the loss of MSP2-specific T cell responses following challenge or in a switch from a Th1 to a Th2 response, IL-10, TGF-β1, and IL-4 levels in supernatants of PBMC cultured for 72 h with MSP2 were determined by ELISA. There was no significant increase in IL-10, TGF-β1, or IL-4 production by PBMC obtained at the peak of infection compared with PBMC obtained before challenge (Tables V and VI). In fact, there was significantly less IL-10 produced by PBMC obtained at peak rickettsemia.

Effect of mixing responding and nonresponding PBMC

To determine whether the MSP2-nonresponsive cells from the peak of rickettsemia contained a population of cells that were suppressive, proliferation assays were performed using a fixed number of responding cells obtained before peak rickettsemia mixed with increasing numbers of nonresponding cells obtained at or after peak rickettsemia (Fig. 5). No significant inhibition of PBMC responses was observed when cells from four different animals were tested in repeated assays. The results for animals 71 and 82 are shown in Fig. 5.

Positively selected CD4$^+$ T cells from peak rickettsemia do not respond to Ag cultured with APC from naive donors

To address the possibility that the inability to detect a CD4$^+$ T cell response following challenge was caused by dysfunctional APC, CD4$^+$ T cells were positively selected from PBMC of two animals (71 and 76) cryopreserved at time points when the cells responded or at peak rickettsemia when no response was detected. PBMC were stimulated with Ag in the presence of MHC class II DRB3 homozygous and half-matched APC from *A. marginale* naive donor cattle. CD4$^+$ T cells obtained at time points where responses to Ag were previously observed had strong and significant proliferative responses to *A. marginale* and MSP2 in the presence of APC from naive donors (Table VII). In contrast, CD4$^+$ T cells obtained at the peak of infection had undetectable proliferative

Table V. *Comparison of cytokines IL-10, TGF-β1, and IL-4 in culture supernatants from responding and nonresponding PBMC stimulated with MSP2: the mean cytokine concentration + 1 SD in supernatants of PBMC from the animals*[a]

			IL-12 Group								CpG Group							
			Animal 71		Animal 75		Animal 76		Animal 82		Animal 78		Animal 79		Animal 81		Animal 87	
Cytokine[b]	cRPMI[c]	PMA[d]	Prepeak	Peak	Prepeak	Peak	Prepeak	Peak	Prepeak	Peak	Prepeak	Peak	Prepeak	Peak	Prepeak	Peak	Prepeak	Peak
IL-10	0	225	43	9	23	5	22	0	8	9	0	8	52	0	13	6	0	3
U/ml (SD)	(0)	(24)	(3)	(1)	(4)	(7)	(3)	(0)	(0)	(13)	(0)	(4)	(4)	(0)	(0)	(0)	(0)	(4)
TGF-β	1171	2436	364	1412	1021	642	1217	3165	1092	1400	1885	1373	2015	2723	1575	702	1421	1516
pg/ml (SD)	(167)	(184)	(226)	(8)	(1449)	(142)	(1105)	(42)	(50)	(1080)	(706)	(605)	(252)	(883)	(958)	(227)	(420)	(1345)
IL-4	116	402	104	28	60	187	344	384	99	349	177	286	386	357	166	147	180	258
pg/ml (SD)	(15)	(99)	(102)	(5)	(10)	(25)	(33)	(27)	(55)	(38)	(9)	(30)	(18)	(86)	(1)	(5)	(7)	(6)

[a] Cryopreserved PBMC from MSP2 vaccinates prior to (pre) or at peak rickettsemia were cultured for 72 h with 10 μg/ml MSP2.
[b] Optimal supernatant dilutions are shown, which were 1/2 for IL-10, 1/16 for TGF-β1, and 1/10 for IL-4.
[c] Negative controls are supernatants from PBMC cultured with complete RPMI 1640 (cRPMI) alone.
[d] Positive controls are supernatants from PBMC from a naive animal stimulated with PMA plus ionomycyin for 72 h.

responses to Ag, but did proliferate to TCGF, as observed when autologous APC were used to present Ag.

Inhibition of lymphocyte viability and proliferation by A. marginale *in vitro*

One possible explanation for the loss of *A. marginale* MSP2-specific responses following challenge is that the high level of rickettsemia resulted in deletion of specific cells. We had observed that concentrations of *A. marginale* Ag (e.g., >25 μg/ml) were often inhibitory when T lymphocyte proliferation assays were performed (33). To determine the effect of *A. marginale* on lymphocyte viability and proliferation to TCGF in vitro, purified initial bodies ranging from the equivalent of 3×10^6 to 5×10^7 organisms/ml (5–80 μg/ml protein) were cultured with PBMC from a naive animal in the presence of 10% TCGF. *A. marginale* inhibited both cell viability and proliferation to TCGF in a dose-dependent manner (Fig. 6).

Discussion

The results of this study do not support our hypothesis that challenge with *A. marginale* expressing MSP2 variants to which the animals had been previously exposed would stimulate strong anamnestic $CD4^+$ T cell and IgG responses directed against conserved and variant-specific epitopes that would be associated with variant-specific organism clearance. In contrast, analysis of the immune response upon challenge demonstrates a newly discovered modulation whereby Ag-specific T cell responsiveness induced by vaccination is lost upon rickettsial challenge.

Table VI. *Comparison of cytokines IL-10, TGF-β1, and IL-4 in culture supernatants from responding and nonresponding PBMC stimulated with MSP2: average responses from all animals*[a]

Cytokine	Average	SD	*t* test (p =)
IL-10			
Pre	20	19	0.04
Peak	5	4	
TGF-β			
Pre	1324	527	0.20
Peak	1617	892	
IL-4			
Pre	190	117	0.07
Peak	250	122	

[a] Amount of cytokine is described in Table V. The responses from all immunized animals were averaged and tested for statistical significance using a paired two-tailed *t* test. *p* values are shown.

Sequencing of *msp2* transcripts from the blood of cattle obtained during ascending and peak rickettsemia ruled out the possibility that acute rickettsemia resulted from selective expansion either of organisms expressing variants of MSP2 that constituted a minor population in the challenge inoculum, or of organisms expressing novel *msp2* sequences. This indicates that either the MSP2-specific immune response induced by immunization or the recall response elicited by the challenge was insufficient to effect clearance.

The lack of strong recall T cell responses to MSP2 following *A. marginale* infection may be related to the uniformly dramatic loss of MSP2-specific $CD4^+$ T cell responses that occurred in all animals concurrently with development of measurable rickettsemia. However, the sustained Th cell response for the first 3 wk following challenge was apparently sufficient to stimulate a boost in IgG production. This infection-mediated immune modulation of a strong $CD4^+$ T cell response directed against multiple antigenic epitopes (25) has not been previously described for any rickettsial pathogen. However, in other persistent pathogen infection models, anergy induced by altered peptide ligand antagonism (41–43), or by T regulatory cells producing either TGF-β1 or IL-10 (37, 44–49), has been shown to play a role in down-regulating T cell responses. Although antigenically variant MSP2 epitopes could potentially act as antagonistic peptides, previous studies did not show the ability of naturally occurring variant epitopes to cause anergy of MSP2-specific T cell lines or clones specific for the agonistic MSP2 variant (10). Furthermore, the disappearance of T cell responses to conserved MSP2 epitopes, as well as variable MSP2 epitopes, argues against antigenic variation in MSP2 as a reason for the abrupt loss of T cell responsiveness.

To address the possibility that *A. marginale* infection induced a T regulatory cell response, experiments were performed to determine changes in the percentage of $CD25^+CD4^+$ T cells during the course of infection, to examine IL-10 and TGF-β1 production by responding and nonresponding cells, to detect the presence of a population of suppressive cells in peripheral blood by mixing responding and nonresponding cells, and to test positively selected $CD4^+$ T cells. Although our results do not support the role of T regulatory cells in the dramatic loss of MSP2-specific $CD4^+$ T cell immune responses, their role cannot be definitively ruled out, because these cells have not been phenotypically characterized in cattle. We were similarly unable to demonstrate a shift from a dominant IFN-γ Th1 response (28) response to an IL-4 dominant response following challenge.

To test the possibility that infection impaired APC to present *A. marginale* Ag to $CD4^+$ T cells, positively selected $CD4^+$ T cells

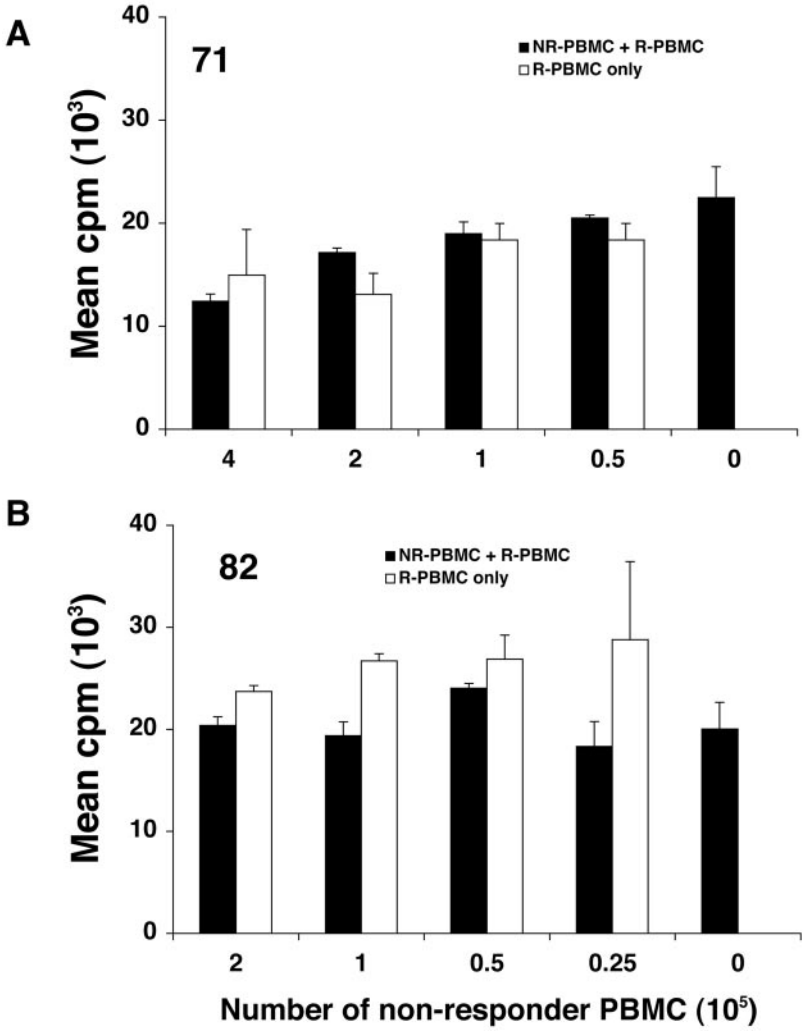

FIGURE 5. Mixing assays of nonresponding and responding PBMC stimulated with 5 μg/ml MSP2. Nonresponding PBMC (NR-PBMC) obtained from animals during peak rickettsemia were cultured at ratios of 4:1, 2:1, 1:1, 0.5:1, or 0:1 with a constant number of responding PBMC (R-PBMC) obtained from animals before peak rickettsemia. Responding PBMC from animal 71 were cultured at 1×10^5 cells/well (*A*) and responding PBMC from animal 82 were cultured at 0.5×10^5 cells/well (*B*).

were cultured with Ag in the presence of class II-compatible APC from noninfected donors. However, T cells obtained at the peak of infection were still unable to respond to Ag, ruling out dysfunctional APC as the reason for the sudden loss of response.

Sheep and dogs infected with *Anaplasma phagocytophilum* develop a transient immunosuppression defined by leukopenia (reduced numbers of T lymphocytes and neutrophils) and an increased susceptibility to other infectious organisms (50–52). *A. phagocytophilum* infects neutrophils and alters neutrophil function (53–57), which may explain the transient generalized immune suppression. However, a similar mechanism of generalized immune suppression by *A. marginale* is unlikely for the following reasons: 1) this pathogen infects erythrocytes and not neutrophils, 2) the response to unrelated clostridial Ags was not severely impaired during acute infection, and 3) increased susceptibility to unrelated or opportunistic infections has not been reported for cattle with anaplasmosis.

The unsubstantiated role of T regulatory cells in the disappearance of the MSP2-specific memory T cell response, the lack of evidence for altered Ag presentation, and the Ag-specific nature of

Table VII. *CD4$^+$ T cells obtained at the peak of rickettsemia fail to proliferate to* A. marginale *MSP2 in the presence of normal APC*

	Proliferation (mean cpm ± 1 SD) of CD4$^+$ T Cells Obtained from[a]			
	Animal 71 (*DRB3 8/14*)		Animal 76 (*DRB3 22/21*)	
Antigen	Prepeak	Peak	Prechallenge	Peak
Medium	762 ± 52	133 ± 27	1,302 ± 497	354 ± 32
TCGF	**103,012 ± 1,038**	**91,768 ± 2,276**	**78,037 ± 5,267**	**16,190 ± 1,647**
URBC	849 ± 726	85 ± 39	1,363 ± 636	323 ± 119
A. marginale	**56,507 ± 2,354**	29 ± 22	**10,544 ± 753**	132 ± 51
MSP2	**60,906 ± 4,724**	48 ± 28	**26,174 ± 7,580**	322 ± 224

[a] CD4$^+$ T cells were obtained by positive selection from PBMC from animals 71 and 76 cryopreserved at 1 wk postchallenge (animal 71) or 2 wk prechallenge (animal 76) or at peak rickettsemia and cultured with APC from MHC class II homozygous and half-matched normal donors. For 71 T cells, APC from animal 98B61 (*DRB3 8/8*) were used, and for 76 T cells, APC from animal 201 (*DRB3 22/22*) were used. Cells were cultured with medium, 10% TCGF, or 10 μg/ml the indicated Ags, and results are presented as the mean cpm ± 1 SD of triplicate cultures. Numbers in boldface type are significantly greater than those for URBC or medium controls ($p < 0.01$).

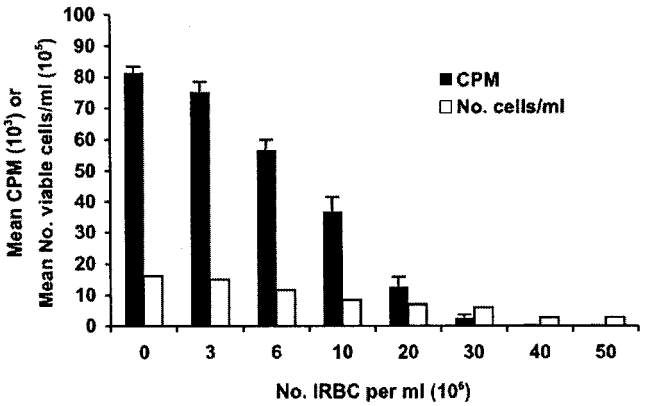

FIGURE 6. Inhibition of lymphocyte viability and proliferation by *A. marginale*. Purified *A. marginale* initial bodies ranging in concentration from 3×10^6 to 5×10^7/ml were cultured for 3 days with 2×10^5 PBMC/well plus 10% TCGF. Data are presented as the mean number of viable cells/milliliter of pooled triplicate wells or the mean cpm + 1 SD of triplicate cultures.

the immune suppression suggest an alternative mechanism for the loss of T cell responsiveness. One potential mechanism is peripheral T cell deletion that could occur via activation-induced cell death (AICD) following organism challenge (58). During primary HIV infection, naturally induced HIV-specific $CD8^+$ T cell clones with defined TCR Vβ usage were shown to rapidly disappear, independent of changes in the viral epitopes recognized (59). An unrelated study reported in vivo elimination of Ag-specific Th1 cells, obtained from TCR transgenic mice that were adoptively transferred to normal mice, following i.v. challenge with the Ag cytochrome *c* 1–2 mo later (60). The Ag-specific memory T cells became rapidly activated in vivo upon Ag administration, but by day 8 following Ag challenge, declined to barely detectable numbers and remained depressed or anergic for 3 mo. The authors concluded that Ag challenge of resting Th1 $CD4^+$ T cells led to transient activation followed by cell depletion. In our studies, *A. marginale* was administered i.v. and the infection took ~5 wk to reach peak levels in peripheral blood. Thus, for the first 3 wk following challenge, recall T cell responses remained at prechallenge levels, but were completely undetectable at the peak of infection, 2 wk later. We therefore examined $CD4^+$ T cell IFN-γ ELISPOT responses in six immunized cattle at ~1 wk before the peak of infection was reached (29 or 31 DPI), and observed weakly positive responses in two animals and undetectable responses in four animals (data not shown). These results are consistent with Ag-induced AICD. Furthermore, *A. marginale* inhibited, in a dose-dependent manner, proliferation of lymphocytes that paralleled a loss in cell viability. A reduction in the response to TCGF from >50 to 100% was observed at organism concentrations equivalent to those observed at peak levels of rickettsemia in vivo following challenge, which ranged from 1×10^7 to 8×10^8 organisms per milliliter of blood (Fig. 2). However, the significance of these in vitro results to the in vivo infection is not clear, because *A. marginale* is generally intraerythrocytic and the nature of the interaction of infected erythrocytes with T cells is unknown. Nevertheless, these results are also consistent with a loss in immune responsiveness as a consequence of increasing Ag dose in vivo, and a mechanism of AICD.

In conclusion, we hypothesize that MSP2-specific memory T cells were deleted or decreased to undetectable numbers in animals following infection with *A. marginale*. Our data indicate a newly discovered immune modulation whereby Ag-specific T cell responsiveness is lost upon rickettsial challenge. MSP-2-specific T cells may be deleted as a consequence of high levels of Ag occurring during ascending rickettsemia, and the number of MSP2-specific T cells may remain depressed as a result of the chronic low antigenic exposure during persistent infection. Consistent with this is our inability to detect $CD4^+$ T cell responses in nonimmunized cattle following either i.v. or tick-transmitted *A. marginale* challenge for up to 3 mo postinfection (this study and our unpublished observations). Additional experiments using MHC class II tetramers to track the fate of epitope-specific T cells in immunized and control cattle during acute and chronic anaplasmosis should clarify the mechanism for the loss of Ag-specific T cell responses following *A. marginale* infection and determine whether a similar immune modulation occurs during infection of nonvaccinated cattle.

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Disclosures

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